Journal of Physiology (1993), **468**, *pp.* 1–10
With 4 figures
Printed in Great Britain

THE RELATION BETWEEN STIMULUS AND RESPONSE IN OLFACTORY RECEPTOR CELLS OF THE TIGER SALAMANDER

BY STUART FIRESTEIN*, CRISTIANA PICCO AND ANNA MENINI†

*From the Istituto di Cibernetica e Biofisica, Consiglio Nazionale delle Ricerche, Via Dodecaneso 33, 16146 Genova, Italy and the *Section of Neurobiology, Yale School of Medicine, 333 Cedar Street, New Haven, CT 06510, USA*

(*Received 24 March 1993*)

SUMMARY

1. Olfactory receptor cells were isolated from the adult tiger salamander *Ambystoma tigrinum* and the current in response to odorant stimuli was measured with the whole-cell voltage-clamp technique while odorants at known concentrations were rapidly applied for controlled exposure times.

2. Three odorants, cineole, isoamyl acetate and acetophenone, were first applied at 5×10^{-4} M. Out of forty-nine cells tested, 53% responded to one odorant only, 22% to two odorants and 25% to all three odorants.

3. The amplitude of the current in response to a given odorant concentration was found to be dependent on the duration of the odorant stimulus and reached a saturating peak value at 1·2 s of stimulus duration.

4. The current measured at the peak of the response for odorant steps of 1·2 s as a function of odorant concentration was well described by the Hill equation for the three odorants with Hill coefficients higher than 1 and $K_{\frac{1}{2}}$ (odorant concentration needed to activate half the maximal current) ranging from 3×10^{-6} to 9×10^{-5} M.

5. It is concluded that olfactory receptor cells are broadly tuned and have a low apparent affinity for odorants, integrate stimulus information over time, and have a narrow dynamic range.

INTRODUCTION

The remarkable ability of the olfactory system to detect and discriminate among thousands of different odorants begins with a transduction mechanism that now appears to be homologous to that employed in visual, hormonal and neurotransmitter signalling (Lamb & Pugh, 1992). In olfactory receptor cells a large family of odorant receptors (Buck & Axel, 1991) coupled to GTP-binding proteins activates a second messenger pathway leading to the generation of cAMP, which directly gates ion channels in the ciliary membrane (Nakamura & Gold, 1987; Firestein, Zufall & Shepherd, 1991). The odorant-stimulated membrane current can be measured with the whole-cell patch-clamp technique in isolated olfactory receptor cells cleaned of mucus (Firestein & Werblin, 1989; Kurahashi, 1989), and provides a measure of the amplitude and time course of the cellular response.

† To whom correspondence should be addressed.

MS 2229

The relation between a stimulus impinging on a receptor cell and the consequent response of the cell itself is a fundamental quantitative measure of signal transduction systems. Unfortunately these data are not currently available for olfactory receptor cells, due primarily to the difficulties associated with accurate and controlled stimulus delivery. Even for isolated cell preparations, where tissue factors are no longer an obstacle, diffusion effects on pressure-ejected aqueous odorant solutions means that the intensity and time course of the stimulus at the receptive membrane can, at best, only be estimated (Firestein & Werblin, 1989; Firestein, Shepherd & Werblin, 1990). This ambiguity of the stimulus input makes an unequivocal interpretation of the cellular response difficult.

We have sought to solve this problem by using a stimulus delivery system similar to the one used to exchange extracellular ion solutions around intact photoreceptor cells (Hodgkin, McNaughton & Nunn, 1985; Menini, Rispoli & Torre, 1988). With this perfusion system an isolated olfactory receptor cell could be completely immersed in a solution containing a known concentration of odorant for controlled exposure times. We measured the currents elicited by three odorants, cineole, isoamyl acetate and acetophenone, at concentrations varying over three orders of magnitude.The data show an olfactory receptor cell that is often capable of recognizing more than one odorant, integrates stimulus information over time, operates at relatively high concentrations, and has a narrow dynamic range.

Preliminary results have been published in abstract form (Firestein, Picco, Spadavecchia & Menini, 1992).

METHODS

Preparation and recording

Land-phase adult salamanders *Ambystoma tigrinum* were decapitated and pithed, and olfactory receptor cells were mechanically isolated, without the use of enzymes, from the nasal epithelium of the animals, as described by Firestein *et al.* (1990). Currents were measured with the Axopatch 1D patch-clamp amplifier (Axon Instruments, USA) in the whole-cell voltage-clamp mode at a holding potential of -55 mV. Patch pipettes were fabricated from Corning 5072 glass and resistances were 4–6 MΩ when filled with internal solution. Currents were filtered at 1 kHz with an 8-pole Bessel low-pass filter, sampled at 2 kHz and stored directly on the hard disk of an IBM PC 486 computer using pCLAMP software (Axon Instruments, Foster City, CA, USA).

Solutions

The normal Ringer solution contained (mM): NaCl, 115; KCl, 2·5; $CaCl_2$, 1; $MgCl_2$, 1·5; Hepes hemisodium, 9; pH 7·5 (total Na^+ = 119·5). A 0·5 M stock solution of each odorant, acetophenone, isoamyl acetate and cineole (from Sigma, St Louis, MO, USA), was prepared in dimethyl sulphoxide (DMSO, from Sigma) and diluted in the Ringer solution to appropriate concentrations. DMSO was never present at more than 0·2%, and had no effect on olfactory cells by itself. The intracellular pipette solution contained (mM): CsCl, 120; $MgCl_2$, 2; $CaCl_2$, 1; EGTA, 2; Hepes, 4; ATP, 1; GTP, 0·1; pH 7·5.

Stimulus delivery

Rapid solution changes were obtained with a perfusion system similar to that described by Hodgkin *et al.* (1985) and Menini *et al.* (1988). Four different solutions were flowing, side by side, into four parallel tubes at the back of the recording chamber. An isolated cell, firmly attached at the tip of a patch pipette, was first placed in front of one pipe in a continuously flowing Ringer solution, and then rapidly moved in front of one of the adjacent pipes, where an odorant–Ringer solution was flowing. Movement of the perfusion chamber was accomplished by a stepping motor under computer control allowing forward and backward movements and exposure times to the

stimulus of variable duration. The time required for changing solution was 30–50 ms, as estimated from the change in current of a cell moved from Ringer solution to 20 mM KCl solution (see upper trace in Fig. 2*A*). A single cell could be exposed to any of forty-eight different solutions. At least 60 s elapsed between stimulus presentations.

RESULTS

Specificity of cells for different odorants

The response of olfactory receptor cells to a mixture of acetophenone, isoamyl acetate and cineole, each present at a concentration of 7×10^{-5} M, was studied in 181 cells and it was found that ninety-seven cells (54%) responded, always with an inward current whose maximal amplitude varied from 50 to 1500 pA. Forty-nine of the responding cells were subsequently tested with individual components of the mixture at 5×10^{-4} M: twelve cells (25%) responded to all three odorants, cineole (Ci), acetophenone (Ac), and isoamyl acetate (Am); twenty-six cells (53%) responded only to one odorant:

$$n_{\mathrm{Ci}} = 21, n_{\mathrm{Ac}} = 2, n_{\mathrm{Am}} = 3, \tag{1}$$

and eleven cells (22%) responded to two odorants:

$$n_{\mathrm{Ci,Ac}} = 5, n_{\mathrm{Ci,Am}} = 2, n_{\mathrm{Ac,Am}} = 4. \tag{2}$$

By a wide margin cineole was the preferred odorant in our sample: forty cells, 82%, responded at least to cineole and twenty-one cells, 43%, to cineole only. However, even within this limited sample, we found cells that responded to every combination of the three odorants, with about half (47%) of the cells responding to more than one odorant.

Figure 1 shows recordings in three different cells responding to: all three odorants (upper row), cineole only (middle row), acetophenone and isoamyl acetate (bottom row). These examples are indicative not only of the variety of odorants a cell may respond to, but also of the variability in sensitivity to different odorants, as suggested by the different magnitudes of a cell's response to equal concentrations (5×10^{-4} M) of various odorants.

Dependence of response on stimulus durations

Olfactory receptor cells were stimulated by odorants at different concentrations for exposure times varying from 0·5 to 1·5 s. A family of responses elicited by 3×10^{-5} M cineole at various step durations is illustrated in Fig. 2*A*. Top traces show the time course of the stimulus obtained as described in the Methods section. The amplitude of the current induced by 3×10^{-5} M cineole increased with step duration, reaching a maximum with steps of about 1·2 s, whereas the onset kinetics was unchanged. The same cell was also tested at 2×10^{-5} and 5×10^{-5} M cineole and the peak currents, normalized to the values obtained with steps of 1·2 s at each concentration, were plotted *versus* step duration in Fig. 2*B*. At each concentration the peak response reached a saturating value at about 1·2 s, and longer stimulus durations did not produce any further increase in response amplitude. The relation between the peak amplitude of the odorant-induced current and the stimulus duration was also concentration dependent, being steeper for lower concentrations. In the extreme we found odorant-sensitive cells that failed to produce a measurable

response at low concentrations if the step duration was less than 0·8 s. Similar results were obtained at various concentrations of the three odorants in five other cells. In the experiments presented in Figs 1, 3 and 4 we have used stimulus durations of 1·2 s and the dose–response relations are presented as peak response *versus* odorant concentration.

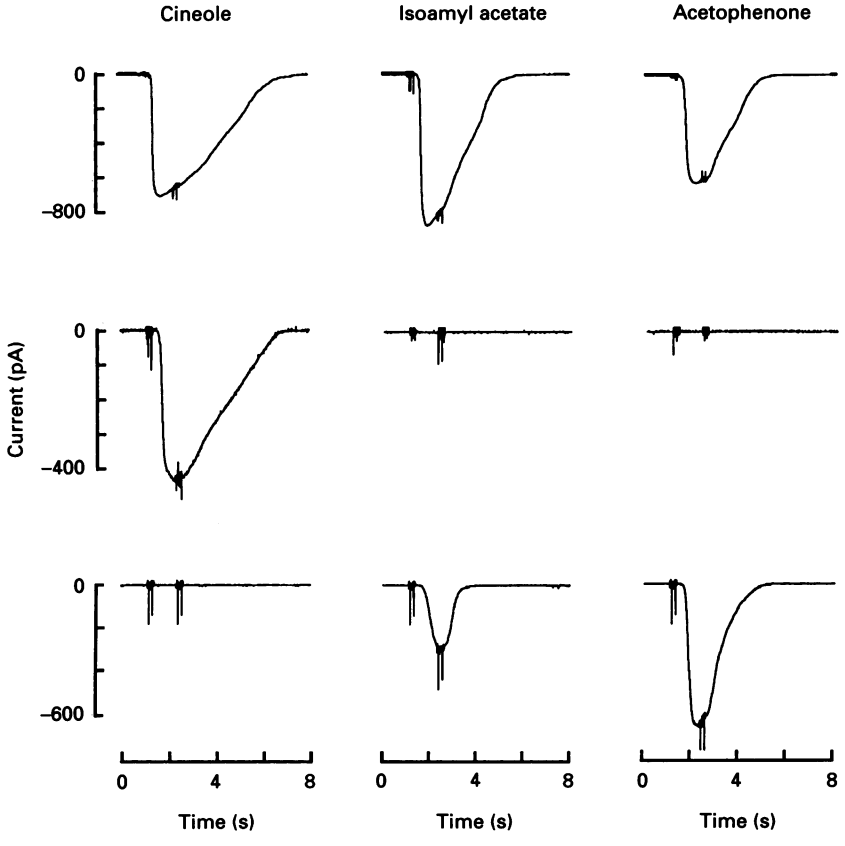

Fig. 1. Current responses to the indicated odorants at 5×10^{-4} M applied for 1·2 s in three different cells responding to all three odorants (upper row), cineole only (middle row), acetophenone and isoamyl acetate (bottom row). An electrical artifact, caused by the stepping motor and indicative of the onset and offset of the stimulus, was present in each trace. Holding potential (V_h) was −55 mV.

Dose–response relations for individual odorants

Currents elicited by 1·2 s step exposures to individual odorant stimuli at concentrations ranging from 5×10^{-7} to 1×10^{-3} M were measured. Figure 3 shows results for acetophenone (*A*), isoamyl acetate (*B*), and cineole (*C*), in different cells. At the top left the stimulus timing trace, obtained as in Fig. 2*A*, is shown. Families of currents recorded in different cells are shown for each odorant (panel *A*). After a latency lasting up to a few hundred milliseconds, the odorant-stimulated currents rose and fell with a slow time course, reaching a peak after at least 0·5 s and decaying

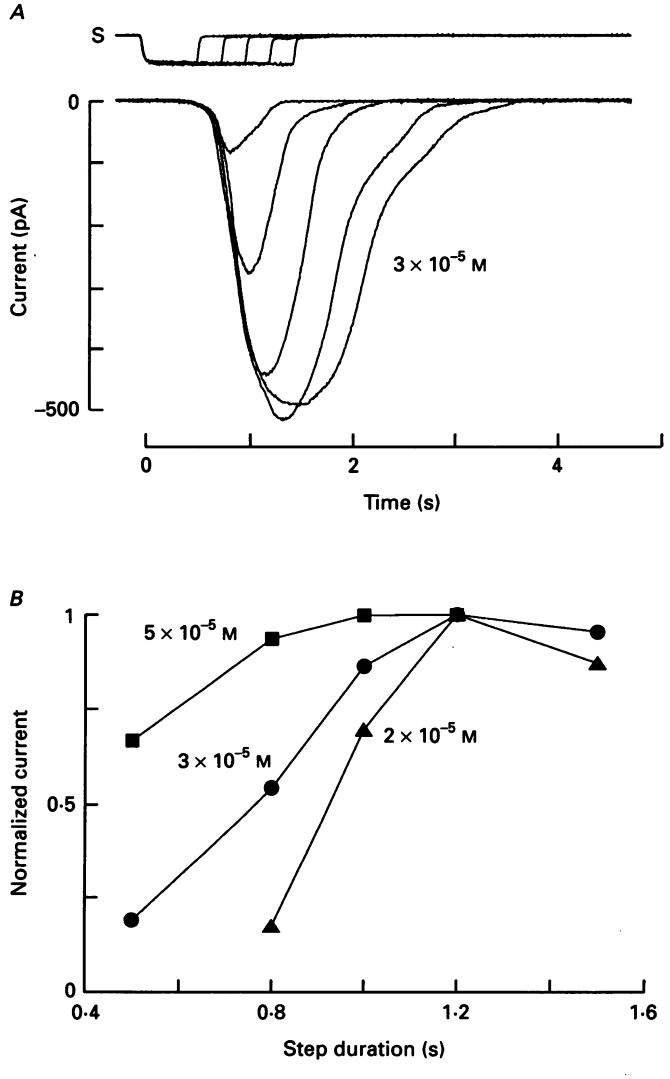

Fig. 2. *A*, family of responses elicited by 3×10^{-5} M cineole for step durations of 0·5, 0·8, 1·0, 1·2 or 1·5 s. The time course of the stimuli, labelled S and shown at the top, was represented by the responses of the same cell to 20 mM KCl instead of odorant–Ringer solution for the same step durations. The solution change was completed in 30 ms and the current in response to KCl remained stable throughout the step duration indicating that the stimulus concentration was stable. *B*, peak currents at different cineole concentrations: 2×10^{-5} M (▲), 3×10^{-5} M (●) or 5×10^{-5} M (■). Current amplitudes were normalized to the peak current measured at each concentration for a stimulus step of 1·2 s, and were plotted *versus* step durations. $V_h = -55$ mV.

back to baseline over a time course that sometimes required 10 s or more. The latency and rate of rise of the response were concentration dependent, becoming shorter and faster, respectively, with increasing stimulus concentration.

Although the responses in the three examples shown here possessed slightly

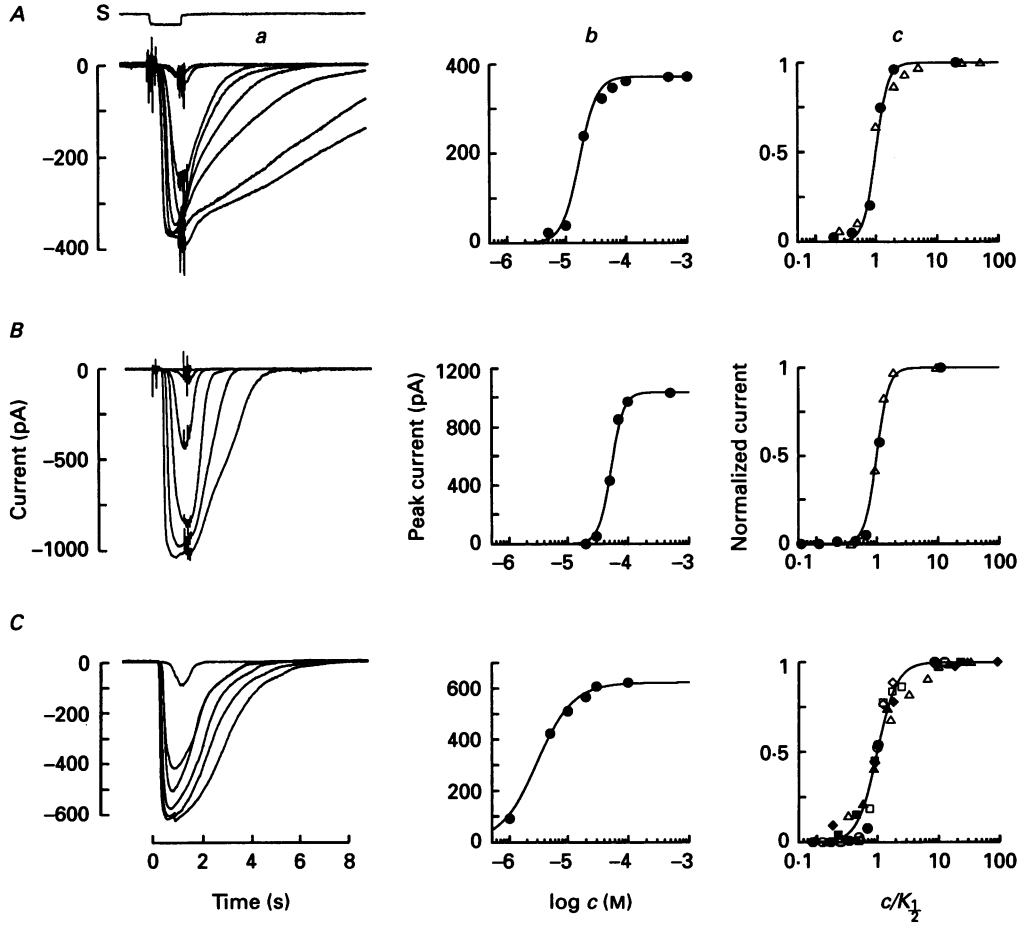

Fig. 3. Families of responses to different concentrations of acetophenone (*Aa*), isoamyl acetate (*Ba*) or cineole (*Ca*) in three cells. The top trace, labelled S, was the response of one cell to 20 mM KCl and represents the stimulus time course, as in Fig. 2*A*. Each cell was sequentially placed in one of a series of solutions with increasing concentrations of odorant for 1·2 s. In *Aa* and *Ba* an electrical artifact caused by the stepping motor was present, whereas it was absent in *Ca*. $V_h = -55$ mV. Panel *b* shows dose–response curves plotted as the peak current *versus* odorant concentration from the experiments shown in panel *a*. The continuous lines were the best fit of eqn (3) to the data with the following values: $I_{p,max} = 372$ pA, $K_{\frac{1}{2}} = 1{\cdot}7 \times 10^{-5}$ M, $n = 3$ for acetophenone (*Ab*); $I_{p,max} = 1034$ pA, $K_{\frac{1}{2}} = 5{\cdot}3 \times 10^{-5}$ M, $n = 4{\cdot}2$ for isoamyl acetate (*Bb*); $I_{p,max} = 623$ pA, $K_{\frac{1}{2}} = 3 \times 10^{-6}$ M, $n = 1{\cdot}4$ for cineole (*Cb*). Panel *c* shows dose–response curves from collected data. Both axes are normalized values. The ordinate (response) is the peak current normalized to the maximal peak current elicited in each cell and the abscissa is the concentration normalized to the $K_{\frac{1}{2}}$ value in each cell. Thus the $K_{\frac{1}{2}}$ for all the data sets is unity. Maximal peak currents and $K_{\frac{1}{2}}$ ranged, respectively, from 297 to 1018 pA and from 3×10^{-6} to 9×10^{-5} M. Continuous lines were the best fit of eqn (3) to the data with $n = 4{\cdot}3$ for acetophenone (*Ac*), $n = 4{\cdot}4$ for isoamyl acetate (*Bc*), and $n = 2{\cdot}7$ for cineole (*Cc*). Best fits were obtained with the Levenberg–Marquardt algorithm.

different kinetics, this appeared to reflect normal between-cell variability. We did not detect any differences in the response kinetics that could be reliably correlated with different odorants.

The response profile could be quantified by constructing dose–response relationships. For each cell the peak current was plotted *versus* the concentration of

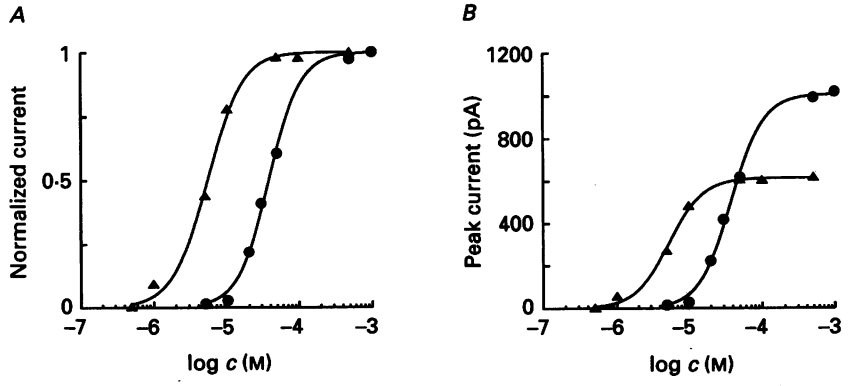

Fig. 4. Dose–response plots of the response to cineole in two different cells are shown as normalized currents in *A*, and as absolute values in *B*. $I_{p,max}$, $K_{\frac{1}{2}}$ and n values for each cell were 618 pA, $5{\cdot}4 \times 10^{-6}$ M, 1·8 (▲) and 1018 pA, $3{\cdot}8 \times 10^{-5}$ M, 1·9 (●), respectively.

odorant at the receptive membrane (Fig. 3*Ab*, *Bb*, and *Cb*). The data were well fitted by the Hill equation:

$$I_p = I_{p,max} c^n / (c^n + K_{\frac{1}{2}}^n) \quad (3)$$

where I_p is the peak response, $I_{p,max}$ the maximal peak response, c the odorant concentration, $K_{\frac{1}{2}}$ the odorant concentration necessary to activate half of the maximal current, and n is the Hill coefficient. The values for n and $K_{\frac{1}{2}}$ were, respectively, 3 and $1{\cdot}7 \times 10^{-5}$ M for acetophenone, 4·2 and $5{\cdot}3 \times 10^{-5}$ M for isoamyl acetate, 1·4 and 3×10^{-6} M for cineole. The Hill coefficients required to fit these data were always greater than 1, indicating a narrow dynamic range. This is brought out more fully in panel *c* of Fig. 3 where data from several cells were combined and plotted, for each odorant, as normalized peak currents ($I_p/I_{p,max}$) *versus* normalized odorant concentration ($c/K_{\frac{1}{2}}$). Continuous lines were the best fit to the collected data of eqn (3) with Hill coefficients of 4·3 for acetophenone (2 cells), 4·4 for isoamyl acetate (2 cells) and 2·7 for cineole (8 cells). It is likely that the higher values for acetophenone and isoamyl acetate are due to the smaller sample sizes for these two odorants, two cells each, and that the value for cineole, from eight cells, is more reliable. In all cases, however, the Hill coefficient was greater than 1, resulting in steep dose–response curves with the 10–90% response range covering only one logarithmic unit or less of concentration change.

Variability of the apparent affinity for the same odorant in different cells

Figure 4*A* shows normalized dose–response relations for cineole, in two different cells: the Hill coefficients from fits of eqn (3) were very similar, 1·8 and 1·9, whereas $K_{\frac{1}{2}}$ values were $5{\cdot}4 \times 10^{-6}$ M and $3{\cdot}8 \times 10^{-5}$ M. The separation in response ranges of

nearly a logarithmic unit was significant given that the entire response range for either cell covered barely a logarithmic unit of concentration change. The same two curves were plotted in absolute values in Fig. 4*B* where it can be seen that the maximal responses in the two cells differed by nearly 2-fold. Perhaps importantly the cell with the lower $K_{\frac{1}{2}}$ for cineole gave the smaller maximal response, suggesting that the difference in the $K_{\frac{1}{2}}$ values was not due to cellular factors such as receptor number or coupling efficiency in the second messenger cascade.

DISCUSSION

The rapid application of precise concentrations of odorant stimuli for prescribed durations to isolated olfactory receptor cells allowed us to record responses of individual cells to specific odorants at known concentrations, and from this data, to construct the first quantitative dose–response relationships for three individual odorant stimuli.

Specificity and affinity

The high frequency, 54%, of finding a responsive cell to a mixture composed of only the three odorants acetophenone, isoamyl acetate and cineole, and the high percentage among these cells of responses to more than one odorant, 47%, indicate that olfactory receptor cells are not very selective. The broad tuning is consistent with results obtained in isolated cells with two odorants, limonene and isoamyl acetate (Kurahashi, Kaneko & Shibuya, 1990) and with earlier findings in intact epithelia (Sicard & Holley, 1984). Although broadly tuned, cells none the less also displayed strong specificity; even when tested at high concentrations (0·5–1 mM) of an ineffective odorant, cells could not be induced to respond (see Fig. 1*B* and *C*).

We cannot determine if a cell responding to more than one odorant has receptors highly specific for each odorant, or receptors capable of binding chemically distinct odorants, or both types of receptors. The finding that different cells responding to the same odorant occasionally showed $K_{\frac{1}{2}}$ values differing by as much as an order of magnitude (Fig. 4) suggests the possibility of having different receptors capable of recognizing the same odor. Moreover, the odorant-induced currents recorded in this, and previous studies on isolated cells (Firestein & Werblin, 1989; Firestein *et al.* 1990) showed relatively low apparent affinities (see Fig. 3), which would be consistent with odorant receptors capable of binding multiple ligands. Finally, it has been recently shown that at least one odorant receptor from the rat responds to several odorants with different efficiency, indicating a relatively broad ligand specificity of odorant receptors (Raming *et al.* 1993).

Integration of stimulus information over time

An interesting finding of this study was that, especially at low odorant concentrations, the response of olfactory receptor cells depends not only on the concentration, but also on the duration of the stimulus (Fig. 2), as previously suggested by Firestein *et al.* (1990). This behaviour suggests that the cell not only measures concentration but integrates concentration information over an integration time of about 1·2 s.

Narrow dynamic operating range

A consistent finding across different cells and different odorants was that the dose–response curves were very steep: a concentration change of less than one decade was sufficient to cover the response range between 10 and 90% (Fig. 3). Hill coefficients larger than 1 usually indicate co-operativity. However, since the ionic currents measured here do not arise directly from odorant binding, but are the result of the second messenger cascade, the site, or sites, of co-operative reactions cannot be determined. It should be noted, however, that the activation of the cAMP-gated channel requires the co-operative binding of at least two or three molecules of cAMP (Nakamura & Gold, 1987; Kurahashi, 1990; Zufall, Firestein & Shepherd, 1991; Frings, Lynch & Lindemann, 1992) and that this could at least partially account for the Hill coefficient of the odorant-induced current.

Physiology of the odorant-induced response

These quantitative dose–response measurements clearly depict that vertebrate olfactory receptor cells are often able to detect more than one odorant, integrate stimulus information over a 1·2 s period, have relatively low apparent affinities for their ligands, and operate over narrow dynamic ranges. By comparison photoreceptors and some transmitter/hormone receptors have dose–response curves spanning up to three orders of stimulus magnitude (Baylor, Hodgkin & Lamb, 1974) and typical $K_{\frac{1}{2}}$ values for transmitter/hormone receptors in the submicromolar range (Kobilka, 1992). However, olfactory receptor cells are unique in the large variety of ligands which they can detect. This appears to be accomplished at the expense of signalling stimulus intensity information, which in turn may be recovered at a later stage in processing by integrating inputs from cells with overlapping response ranges.

We thank Vincent Torre and Gordon Shepherd for helpful discussions and comments on the manuscript. This research was supported by the European Economic Community Grant BRA SSS 6961, Progetto Bilaterale CNR, a NATO Collaborative Research Grant, Office of Naval Research (USA), and the National Institutes of Health (NIDCD).

REFERENCES

BAYLOR, D. A., HODGKIN, A. L. & LAMB, T. D. (1974). The electrical response of turtle cones to flashes and steps of light. *Journal of Physiology* **242**, 685–727.

BUCK, L. & AXEL, R. (1991). A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell* **65**, 175–187.

FIRESTEIN, S., PICCO, C., SPADAVECCHIA, L. & MENINI, A. (1992). Dose–response relationships of individual olfactory neurons exposed to known concentrations of odors. *Chemical Senses* **17**, 833–834.

FIRESTEIN, S., SHEPHERD, G. M. & WERBLIN, F. S. (1990). Time course of the membrane current underlying sensory transduction in salamander olfactory receptor neurones. *Journal of Physiology* **430**, 135–158.

FIRESTEIN, S. & WERBLIN, F. (1989). Odor-induced membrane currents in vertebrate-olfactory receptor neurons. *Science* **244**, 79–82.

FIRESTEIN, S., ZUFALL, F. & SHEPHERD, G. M. (1991). Single odor-sensitive channels in olfactory receptor neurons are also gated by cyclic nucleotides. *Journal of Neuroscience* **11**, 3565–3572.

FRINGS, S., LYNCH, J. W. & LINDEMANN, B. (1992). Properties of cyclic nucleotide-gated channels mediating olfactory transduction. *Journal of General Physiology* **100**, 1–11.

HODGKIN, A. L., MCNAUGHTON, P. A. & NUNN, B. J. (1985). The ionic selectivity and calcium dependence of the light-sensitive pathway in toad rods. *Journal of Physiology* **358**, 447–468.

KOBILKA, B. (1992). Adrenergic receptors as models of G protein-coupled receptors. *Annual Review of Neuroscience* **15**, 87–114.

KURAHASHI, T. (1989). Activation by odorants of cation-selective conductance in the olfactory receptor cell isolated from the newt. *Journal of Physiology* **419**, 177–192.

KURAHASHI, T. (1990). The response induced by intracellular cyclic AMP in isolated olfactory receptor cells of the newt. *Journal of Physiology* **430**, 355–371.

KURAHASHI, T., KANEKO, A. & SHIBUYA, T. (1990). Ionic mechanisms of the olfactory transduction studied on isolated receptor cells of the newt. *Neuroscience Research Supplement* **12**, S85–96.

LAMB, T. D. & PUGH, E. N. (1992). G-protein cascades: gain and kinetics. *Trends in Neurosciences* **15**, 291–298.

MENINI, A., RISPOLI, G. & TORRE, V. (1988). The ionic selectivity of the light-sensitive current in isolated rods of the tiger salamander. *Journal of Physiology* **402** 279–300.

NAKAMURA, T. & GOLD, G. H. (1987). A cyclic nucleotide-gated conductance in olfactory receptor cilia. *Nature* **325**, 442–444.

RAMING, K., KRIEGER, J., STROTMANN, J., BOEKHOFF, I., KUBICK, S., BAUMSTARK, C. & BREER, H. (1993). Cloning and expression of odorant receptors. *Nature* **361**, 353–356.

SICARD, G. & HOLLEY, A. (1984). Receptor cell responses to odorants: similarities and differences among odorants. *Brain Research* **292**, 283–296.

ZUFALL, F., FIRESTEIN, S. & SHEPHERD, G. M. (1991). Analysis of single cyclic nucleotide gated channels in olfactory receptor cells. *Journal of Neuroscience* **11**, 3573–3580.